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USAMRICD-TR-99-02

Measure of O^6-Alkylguanine-DNA Alkyltransferase Activity in Normal Human Epidermal Keratinocytes in Culture and Effects of *Bis*-(2-chloroethyl) sulfide on the Activity

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May 1999

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (*Leave blank*)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 1999	January 1997 to September 1998

4. TITLE AND SUBTITLE
Measure of O^6-Alkylguanine-DNA Alkyltransferase Activity in Normal Human Epidermal Keratinocytes in Culture and Effects of *Bis*-(2-chloroethyl)sulfide on the Activity.

5. FUNDING NUMBERS
TC1

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
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10. SPONSORING/MONITORING AGENCY REPORT NUMBER
USAMRICD-TR-99-02

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)

O^6-Alkylguanine-DNA alkyltransferase (AGT) is a DNA repair protein that removes alkyl adducts from O^6-alkylguanine in DNA. AGT may be important in DNA repair following injury induced by *bis*-(2-chloroethyl)sulfide (sulfur mustard, HD), since O^6-alkylguanine is one of the HD alkylation products. One model for HD-induced injury uses normal human epidermal keratinocytes (NHEK) in culture. In this study we measured the levels of AGT in NHEK grown to either 60- 80% or to 100% confluency and then studied the effects of a one-hour exposure to 50, 100, and 300 μM HD on AGT activity. Mean AGT activity in NHEK grown to 60-80% and 100% confluency was 470 ± 404 and 518 ± 737 fmol/mg protein, respectively. In general, AGT activity appeared to increase after exposure to 100 μM HD and decrease with increased confluency and after exposure to 50 and 300 μM HD. However, a two-way analysis of variance for cell confluency and HD concentration showed no significant differences between cell confluencies or the different HD concentrations.

14. SUBJECT TERMS
Sulfur mustard, DNA damage, DNA repair, AGT, normal human epidermal keratinocytes, O^6-methylguanine-DNA methyltransferase

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED

ACKNOWLEDGEMENTS

The authors wish to acknowledge Ms. Theresa Nipwoda for growing the NHEK, Mr. Offie Clark for help with HD exposures, Dr. William Smith for useful discussions and help with HD exposures, and Ms. Robyn Lee for help with the statistical analysis.

INTRODUCTION

Sulfur mustard (HD) is a potent alkylating agent that has a long history of use as a chemical warfare agent. Despite decades of medical research, the mechanism by which HD induces vesication is not known, and no effective antidotes are currently available. The fact that DNA is a major cellular target for HD alkylation has been well documented (Fox and Scott, 1980; Rozmiarek et al., 1973). HD alkylates DNA to form ethylthioethyl adducts to include monofunctional adducts at the O^6 position of guanine (Papirmeister et al., 1991).

O^6-Alkylguanine-DNA alkyltransferase (AGT) is a repair protein that acts by transferring the alkyl group from O^6-alkylguanine to a cysteine residue in its center. The reaction is stoichiometric: each molecule of AGT removes one alkyl group, which becomes covalently bound to the AGT cysteine residue (Brent, 1985; Lindahl et al., 1988; Yarosh, 1985; Pegg and Dolan 1987). The alkylated transferase molecule is then catabolized. The cell's ability to perform this type of repair depends strictly on the level of AGT (Yarosh, 1985; Pegg and Dolan, 1987). The level of AGT varies greatly among species, and different tissues contain different levels of alkyltransferase activity (Gerson et al., 1986).

Previous studies have established that normal human epidermal keratinocytes (NHEK) in culture can serve as useful models for biochemical investigations into the toxicity of HD (Smith et al., 1990). Here we report the AGT activity in cultured NHEK grown to either 60-80% or to 100% confluency, and the effects of a one-hour exposure to 50, 100, and 300 μM HD on the AGT activity.

METHODS

*Sulfur mustard. Bis-(*2-chloroethyl)sulfide (5 μl in 10 ml of Keratinocyte Growth Medium [KGM]) was obtained from the U.S. Army Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, MD).

Cell culture. Primary cultures of adult NHEK (strain 4075) were obtained from Clonetics Corporation (San Diego, CA). Tertiary cultures of NHEK were passed into 150 cm^2 tissue culture flasks and grown until either 60-80% or 100% confluency. At this time 50, 100, or 300 μM HD was added to half the flasks, while the other half of the flasks served as the controls. Seven samples were exposed to 50 μM HD, seven samples to 100 μM HD, and seven samples to 300 μM HD. The flasks were left at room temperature for one hour and then harvested by trypsinization according to the procedure described by Boyce and Ham (1985). The harvested NHEK were collected in a conical tube and placed in a styrofoam box containing dry ice until AGT analysis. The cell counts, determined using trypan blue staining, ranged from $2.0x10^7$ to $4.6x10^7$ cells/culture/sample.

Assay of AGT activity. The activity of AGT in NHEK was measured in the laboratory of Dr. Eileen Dolan at the Division of Hematology-Oncology, the University of Chicago Medical Center (Chicago, Illinois) as previously described (Dolan et al., 1990). Briefly, the NHEK cells were incubated with [^{3}H]methylated DNA, which was prepared by allowing [^{3}H]N-methylnitrosourea to react with calf thymus DNA. The DNA was precipitated then acid hydrolyzed, and the modified bases were separated by reverse-phase HPLC. Protein was determined by the method of Bradford (1976), and the results are expressed as fmol of O^6-methylguanine released from the DNA substrate per mg protein. Each sample was run in

duplicate. The results are reported as the mean ± the standard deviation. The AGT activity in NHEK exposed for one hour to 50, 100, or 300 μM HD is reported as percent change of control where % change = [(control-HD)/control] x 100.

RESULTS

Table I shows the mean AGT activity measured for the cultured NHEK. Mean AGT activity measured in 12 samples of NHEK grown to 60-80% confluency was 470 ± 404 fmol/mg protein. Mean AGT activity measured in 6 samples of NHEK grown to 100% confluency was 518 ± 737 fmol/mg protein. A two-way analysis of variance showed no significant differences between confluency levels. Because there were no significant differences, all AGT activities were grouped together and the mean calculated. The mean AGT activity for the 18 samples was 485 ± 515 fmol/mg protein, which is not significantly different from zero.

Table II shows the percent changes of the control of AGT activity in NHEK, at 60-100% confluency, for the HD-exposed samples. The percent changes of the control of the AGT activity for the 50, 100, and 300 μM HD exposed NHEK were -18 ± 167%, +52 ± 98.1%, and -72 ±86.9%, respectively. A two-way analysis of variance showed no significant differences among the HD exposure concentrations (as compared with the controls). Because there were no significant differences among HD exposure concentrations, the percent changes for all three different concentrations were grouped together and averaged. The mean percent change following exposure of the NHEK to HD is 10.06 ± 88.9%, which is not significantly different from zero.

DISCUSSION

This is the first report of AGT activity in cultured NHEK as well as in NHEK exposed to HD. The AGT activity in 60-80% confluent control NHEK (470 ± 404 fmol/mg protein) is higher than that reported for other human tissues, such as brain (67 ± 40 fmol/mg protein), lung (86 fmol/mg protein), myeloid precursors (126 ± 59 fmol/mg protein), colon (138 ± 78 fmol/mg protein), and small intestine (242 ± 168 fmol/mg protein). It is, however, comparable to that reported for T-lymphocytes (359 ± 72 fmol/mg protein) and liver (485 ± 126 fmol/mg protein) (Gerson et al., 1986).

In general, AGT activity decreased with an increase in confluency. The trends for change in AGT activity in NHEK following a one-hour HD exposure were for an increase in activity after 100 μM HD and a decrease in activity after 50 and 300 μM HD. None of these trends was supported by statistical analyses, which showed no significant differences in AGT activities between NHEK of 60-80% and 100% confluency and no significant differences in AGT activity in unexposed NHEK versus NHEK exposed to 50, 100, or 300 μM HD. While the statistical analyses show that AGT activity is not a function of cell confluency and that a one-hour exposure to 50, 100, and 300 μM HD does not affect AGT activity in NHEK in culture, the general trends do show some correlation indicating that a greater sample population may provide statistical support for the observed trends.

Table 1. Mean O^6-Alkylguanine-DNA Alkyltransferase (AGT) Activity in Normal Human Epidermal Keratinocytes in Culture

Number of NHEK Samples	% Confluency	AGT Activity (fmol/mg protein)
12	60 - 80	470 ± 404
6	100	518 ± 737
18[a]	60 - 100	485 ± 515

[b]The mean AGT activity for the 18 NHEK samples.

Table II. Percent Changes of Control of O^6-Alkylguanine-DNA Alkyltransferase Activity in Normal Human Epidermal Keratinocytes, at 60-100% Confluency, Exposed to Different Sulfur Mustard (HD) Concentrations

Number of Determinations	HD Concentrations (μM)	Percent Change[b]
7	50	-18 ± 167
7	100	+ 52 ± 98.1
7	300	-72 ± 86.9

[b]Percent change = [(control - HD exposed)/control] x 100

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